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Nested-PCR and Uracil-N-Glycosylase-significant approach to prevent amplicon contamination in tuberculosis PCR performing laboratories

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ABSTRACT

In developing nations the frequency in using PCR for the diagnosis of tuberculosis is continuously increasing in the routine diagnostic laboratories. The technique is now being accepted as the assay is being upgraded with the incorporation of additional reagents, chemicals, bio-molecules to remove very common contaminant i.e. amplicons. Study includes 125 CSF specimens processed simultaneously by Nested PCR utilising Uracil-N-Glycosylase enzyme and conventional PCR targeting IS6110. It has been seen that using conventional PCR, the frequency of getting false result due to amplicons were more as compared to nested PCR utilizing UNG which degraded the previous amplicons generated. Nested PCR for tuberculosis detection is a better tool when incorporated with an addition of UNG to prevent amplicon contamination. False positive cases by amplicon contamination can be prevented by UNG and dUTP instead of dTTP, during master mix preparation. The skill set required to adequately treat critically ill patients will also require knowledge of molecular biology for better diagnosis and treatment. Amplicon contamination detection and its prevention is of critical importance where the results interpretations are directly involved with patient's health.

Keywords: Nested PCR, Uracil-N-Glycosylase, Amplicon, Pre-mix, False-positive PCR, Anti-contamination strategies.

INTRODUCTION

The high incidence of Tuberculosis (TB) worldwide necessitates research on developing precise diagnostic methods for specific treatment and management (1). A range of molecular assays for tuberculosis have been developed based on PCR, targeting different sequences of which IS6110 is most commonly used in the detection of *M. Tuberculosis* complex (MTB complex) as it is highly conserved. Different protocols have been demonstrated by various researchers involving numerous chemistries (2, 3). In developing nations, the burden of tuberculosis is more and geographically, the burden of TB is highest in Asia and Africa. India and China together account for almost 40% of the world's TB cases. About 60% of cases are in the South-East Asia and Western Pacific regions. The African Region has 24% of the world's cases and the highest rates of cases and deaths per capita. Nucleic acid molecular techniques have become important diagnostic tools in most of the clinical laboratories (4). PCR contamination is one of the important issues for those laboratories performing forensic procedures and detection of infectious agents. Nucleic acid contamination in molecular diagnostics settings adversely affects laboratory results resulting in deleterious effects on the patient's health (5). There are different sources of PCR contamination, the most significant one is PCR products (amplicon) from previous amplifications, also called as carryover contamination. Due to

excessive amplification efficacy of PCR as well as high sensitivity of these techniques, makes them vulnerable to contamination. PCR has the ability to generate up to 1012 product molecules in a single reaction; the most serious source of contamination. Huge amounts of constantly generation of the amplicons increases the potentiality of contamination which is further aided by the fact that a lot of diagnostic applications require PCR to perform at its highest sensitivity, namely, at the single-molecule level and these circumstances, generates billions of molecules from a single reaction, enough to produce a false-positive result (6,7). Amplicons or the products of the amplification reaction can be from positive quality controls which mainly consist of cloned plasmid or patient DNA, matrix template from patients, contaminated water utilized for reagent preparation. There are no publications on techniques or methods on how to detect contamination routine Tuberculosis PCR performing laboratories. There are a numerous approaches to control PCR contamination, and the degree of severity that is required in a laboratory is frequently determined by the assay being performed. Detection of infectious agents typically demands the most stringent contamination efforts, whereas detection of other targets, such as those from inherited disease, may require less contamination control (8). Regardless of the template to be detected, good laboratory practices should be followed. The choice of method is often dependent on the incidence of amplification of the amplicon and the relative amounts and concentrations of the amplicons created by the PCR. Uracil-DNA-glycosylase, also known as UDG is very effective at destroying PCR amplicons when vigorously used for sample preparation (Longo et al. 1990; Thornton et al. 1992). Briefly, at the step of master mix preparation, dTTP is substituted with dUTP, and UDG is included in the reaction mix. All other reaction components for amplification reaction will remain same. During the extension step in PCR, Taq DNA polymerase substitutes dU for dT in the new growing DNA strand, resulting in the final product, which will contain dU instead of dT in the DNA sequence. Prior to processing any new specimen, it first is exposed to UDG enzyme. If UDG comes across any U-containing DNA strands, the U's are cleaved, leaving the strand with gaps. Following heating in the next PCR, the basic strands fall apart and cannot be amplified (9,10). The use of UDG provides the additional benefit of a hot start by degrading all products of the PCR made earlier to the first full cycle (11, 12). We carried out the present study utilizing conventional PCR for the detection of Mycobacterium tuberculosis in CSF specimens in parallel to Nested PCR using uracil-N-glycosylase (UNG) enzyme in order to check the frequency of amplicon contamination, where both the protocols were continuously monitored with the use of negative controls and internal controls. In both the protocols highly conserved sequence; IS6110 is amplified for the detection of Mycobacterium tuberculosis.

MATERIALS AND METHODS

One hundred and twenty five CSF specimens from different Departments of SMI Hospital, Patel Nagar, Dehradun were considered for the study, which was approved by the Institutional Ethical Committee. All the specimens were subjected parallel for MTB complex detection by Nested PCR using uracil-N-glycosylase (UNG) enzyme in pre-mix targeting IS6110 and another one by conventional PCR using IS6110 gene for MTB complex detection. Both the protocols were subjected with controlled parameters utilizing nuclease free water as negative control where after every three specimens a negative control was processed to check any sort of contamination. Nested PCR was performed utilizing manufacturer protocol (Bangalore genei). During the incubation with UNG, any dUTP-amplicons that was unintentionally carried into the samples destined for the next round of amplification is subjected to enzymatic hydrolysis by UNG. First, the UNG-treated DNA is unstable to the subsequent denaturing temperatures of the next round of PCR and is degraded into smaller fragments, which are inappropriate as templates for amplification. In case of Nested PCR, an amplification product of size 123 bp is indicative of infection with Mycobacterium tuberculosis complex where as the amplification product of internal control DNA is 340 bp. In case of conventional PCR, only 123 base pair product indicates Mycobacterial infection.

RESULTS

Nested TB PCR method gave twenty two positive CSF cases (17.6%) where as eighteen CSF came positive (14.4%) by conventional IS6110TB PCR. It was observed twice that IS6110TB PCR gene got amplified in negative controls in case of conventional PCR. IS6110 target in case of N-PCR showed no amplification in negative controls. Invalid results in conventional IS6110TB PCR based PCR clearly demonstrate the presence of amplicons in pre mix for the PCR set up.

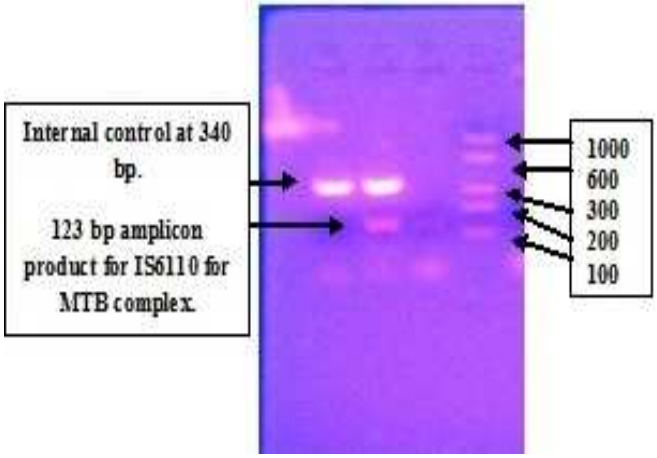

Fig.1. Agarose gel picture with results interpretation: lane 1, well no. 1-Negative specimen, well 2- positive specimen by N-PCR, well 3- positive specimen for MTB complex by IS6110, well 4; DNA ladder for N-PCR.

DISCUSSION AND CONCLUSION

Timely detection of various forms of extra pulmonary tuberculosis is of great importance for the proper treatment and management of the disease. Novel, rapid and cost effectiveness are the basic features of most of the PCR based techniques, but the technologies which can be either bio molecules, chemicals, antibodies or any recombinant proteins incorporating in the assays it to prevent amplicon contamination will be an added advantage. As nested PCR itself increases the sensitivity and specificity of an assay and when UNG is being utilized in pre-mix will make the assay a significant molecular diagnostic tool for Mycobacterium tuberculosis complex detection. Nested PCR for tuberculosis detection is a better tool when incorporated with an addition of UNG to prevent amplicon contamination. False positive cases by amplicon contamination can be prevented by UNG and dUTP instead of dTTP. The skill set required to adequately treat critically ill patients will also require knowledge of molecular biology for better diagnosis and treatment. The foundations of molecular biology and genetics are essential for the understanding of the mechanisms of disease. Correct, novel, significant molecular diagnostic tools are very important for all those laboratories performing routine diagnosis of tuberculosis in PCR based laboratories settings. In addition, particular emphasis should be applied to quality control and quality assurance programs in clinical laboratories which employ any new diagnostic approaches. Amplicon contamination detection and its prevention is of critical importance where the results interpretations are directly involved with patient's health. Acceptance and implementation of PCR in the diagnostic laboratory requires an understanding of its mechanics, meaning of results, the test's limitations, and being able to recognize problems and trouble-shoot them as they arise.

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